

Variation in DNA Methylation Patterns is More Common among Maize Inbreds than among Tissues

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Abstract

Chromatin modifications, such as DNA methylation, can provide heritable, epigenetic regulation of gene expression in the absence of genetic changes. A role for DNA methylation in meiotically stable marking of repetitive elements and other sequences has been demonstrated in plants. Methylation of DNA is also proposed to play a role in development through providing a mitotic memory of gene expression states established during cellular differentiation. We sought to clarify the relative levels of DNA methylation variation among different genotypes and tissues in maize (*Zea mays* L.). We have assessed genomewide DNA methylation patterns in leaf, immature tassel, embryo, and endosperm tissues of two inbred maize lines: B73 and Mo17. There are hundreds of regions of differential methylation present between the two genotypes. In general, the same regions exhibit differential methylation between B73 and Mo17 in each of the tissues that were surveyed. In contrast, there are few examples of tissue-specific DNA methylation variation. Only a subset of regions with tissue-specific variation in DNA methylation show similar patterns in both genotypes of maize and even fewer are associated with altered gene expression levels among the tissues. Our data indicates a limited impact of DNA methylation on developmental gene regulation within maize.

EPIGENETIC VARIATION can result in altered gene expression or phenotype without requiring changes in DNA sequence. Epigenetic information can provide gene regulation during development and differentiation of cells to reinforce the “memory” of transcriptional states. In some cases, epigenetic changes can also be heritable and result in transgenerational memory. Epigenetic marks within the genome are often encoded through chromatin modifications including DNA methylation and histone modifications. Different types of chromatin modifications can have varying functional consequences and there is also evidence that different types of modifications may have varying roles in meiotic and mitotic epigenetic memory. Herein we are focused on the role of DNA methylation in providing epigenetic memory during development (mitotic) and among individuals of different genetic backgrounds (meiotic).

There is fairly strong evidence that DNA methylation patterns can vary among individuals of the same species. There are also many examples of epigenetic variation that generates epialleles resulting in phenotypic variation without sequence changes (Bender and Fink, 1995; Jacobsen and Meyerowitz, 1997; Cubas et al., 1999; Morgan et al., 1999; Chandler et al., 2000; Stokes et al., 2002; Rakyant et al., 2003; Suter et al., 2004; Manning et al., 2006). Genomewide scans in *Arabidopsis thaliana*

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Abbreviations: C(t), threshold cycle; DAP, days after pollination; DMR, differentially methylated region; gDMR, genotype differentially methylated region; GEO, Gene Expression Omnibus; IGV, Integrative Genomics Viewer; IP, immunoprecipitation; MeDIP, methylated DNA immunoprecipitation; qPCR, quantitative real time polymerase chain reaction; RPKM, reads per kilobase exon per million reads; SNP, single nucleotide polymorphism; tDMR, tissue-specific differentially methylated region.

(L.) Heynh. have found DNA methylation differences among ecotypes (Vaughn et al., 2007) and in spontaneous mutant accumulation lines (Becker et al., 2011; Schmitz and Ecker, 2012). There are also examples of variable DNA methylation levels among maize inbreds (Eichten et al., 2011). The variation in DNA methylation patterns among individuals of the same species includes examples that are correlated with genetic changes and those that are not linked to DNA sequence change (Richards, 2006). The genetic changes that are correlated with DNA methylation variation often include transposon insertions or structural rearrangements (Richards, 2006; Girard and Freeling, 1999; Feschotte, 2008; Weil and Martienssen, 2008).

Several groups have also proposed that DNA methylation also plays an important role in direct regulation of genes during development (Bird, 1997; Richards, 1997). There is fairly strong evidence to demonstrate developmental variation in DNA methylation patterns that are correlated with developmental regulation of gene expression in humans (*Homo sapiens sapiens*) (Lister et al., 2009; Hawkins et al., 2010; Franklin et al., 1996; Reik, 2007; Skinner, 2011; Ohtani and Dimmeler, 2011). In addition, several specific cell types or tissues related to reproduction exhibit altered DNA methylation levels in plants (Lauria et al., 2004; Gehring et al., 2009; Hsieh et al., 2009; Zemach et al., 2010). It has been suggested that demethylation in the central cell and vegetative nuclei of the male gametes (Slotkin et al., 2009) is a mechanism to reinforce transposon silencing each generation (Kohler and Weinhofer-Molisch, 2010). Although examples exist of the importance of DNA methylation for proper development in animals and in gametes or endosperm of plants, there is limited evidence that DNA methylation variation contributes to regulatory variation among plant vegetative tissues. The phenotypes observed in *Arabidopsis thaliana* methyltransferase 1 loss-of-function lines include a variety of developmental abnormalities that may suggest a role of DNA methylation in development (Finnegan et al., 1996; Ronemus et al., 1996), although it wasn't clear whether this was due to direct effects of altered DNA methylation patterns. Global DNA methylation levels may change in different vegetative tissues of tomato (*Solanum lycopersicum* L.) (Messeguer et al., 1991). One study suggested a difference in the methylation levels of photosynthesis-related genes during differentiating maize leaf cells (Ngernprasirtsiri et al., 1989). A recent study in *Arabidopsis thaliana* provides evidence that DNA methylation regulates the expression of *WUSCHEL* (Li et al., 2011).

However, there are also several pieces of evidence to suggest somewhat limited changes in DNA methylation during vegetative development. Genomewide profiling of DNA methylation levels in different tissues of rice (*Oryza sativa* L.) (Zemach et al., 2010) identified few examples of differences in DNA methylation levels among vegetative tissues compared to the differences observed between endosperm and other tissues. The analysis DNA methylation patterns in several different tissues

of sorghum [*Sorghum bicolor* (L.) Moench] have shown that the majority of methylation patterns are similar across tissues; however, a small number of tissue-specific differentially methylated regions (tDMRs) were identified that correlate with variable gene expression within their respective tissues (Zhang et al., 2011).

To gain insights into the role of DNA methylation in providing epigenetic memory during development or among individuals, we profiled DNA methylation patterns in two inbred genotypes of maize in four distinct tissues: 14-d leaf, endosperm, tassel, and embryo tissues. Although these complex tissues contain a variety of cell types, we are interested in broad DNA methylation variation across these diverse tissues. Maize provides a unique and robust system to study the role of epigenetic modifications due to the complex organization of interspersed transposons and genes in the maize genome and due to substantial genetic resources (Yu et al., 2008; McMullen et al., 2009; Schnable et al., 2009). Genomewide profiling of methylation patterns between these four tissues show that there are numerous methylation differences between the two genotypes that are observed in all tissues studied. However, there are relatively few differences in DNA methylation levels among tissues. This study shows that few tissue-specific methylation events occur within maize and suggests that the impact of DNA methylation on proper developmental regulation is limited.

Materials and Methods

Plant Materials and DNA Isolation

Seedling leaf tissue was harvested and prepared as described in Eichten et al. (2011). For embryo, tassel, and endosperm collection, plants were grown to maturity in the University of Minnesota Agricultural Research station, Falcon Heights, MN. Endosperm and embryo were harvested from multiple B73 and Mo17 ears 14 d after self-pollination. Multiple endosperms and embryos were pooled from each ear on each date and frozen in liquid N. Immature 15 to 20 cm tassel tissue was harvested from multiple B73 and Mo17 plants and frozen in liquid N. Samples of DNA were isolated using the cetyltrimethylammonium bromide method (Doyle, 1987). Five to 10 micrograms of genomic DNA in 650 to 700 μ L nuclease-free water was sonicated for five 10-s pulses as per the methods of Haun and Springer (2008). Samples were quantified and run on 1.5% agarose gels to verify that DNA was fragmented to 100 to 500 bp.

Array Design and Annotation

Two array platforms were used in this experiment. Endosperm, leaf, and embryo tissues were assayed using a NimbleGen 2.1M feature long oligonucleotide array [GEO (Gene Expression Omnibus) platform GPL13499] as described in Eichten et al. (2011). The analyses in this manuscript focus on a set of ~1.4 million probes from this array that are single copy in the B73 genome and that do not exhibit strong comparative genomic

hybridization variation among B73 and Mo17. Tassel tissue was assayed using a NimbleGen 3x1.4M long oligonucleotide array (GEO platform GPL15621) containing the same subset of 1.4 million probes also found on the 2.1M platform. Partial array replication was performed as outlined in Supplemental Table S1.

Immunoprecipitation of Methylated DNA, Labeling, and Hybridization

Methods were adapted from Eichten et al. (2011). Briefly, methylated DNA was immunoprecipitated with an anti-5-methylcytosine monoclonal antibody from 400 ng sonicated DNA using the Methylated DNA IP (immunoprecipitation) Kit (Zymo Research; catalog no. D5101). For each replication and genotype, whole genome amplification was conducted on 50 to 100 ng IP DNA and also 50 to 100 ng of sonicated DNA (input control) using the Whole Genome Amplification kit (Sigma Aldrich; catalog no. WGA2-50RXN). For each amplified IP input sample, 3 µg amplified DNA were labeled using the NimbleGen Dual-Color Labeling Kit (catalog no. 05223547001) according to the manufacturer's protocol for methylation arrays (Roche NimbleGen Methylation User Guide version 1.0 [Roche NimbleGen, 2011]). Each IP sample was labeled with Cy5 and each input and control sonicated DNA sample was labeled with Cy3. Samples were hybridized to the array for 16 to 20 h at 42°. Slides were washed and scanned according to NimbleGen's protocol for the GenePix4000B or Nimblegen MS200 array scanner. Images were aligned and quantified using NimbleScan software (Roche NimbleGen, 2010) producing raw data reports for each probe on the array.

Normalization and Linear Modeling

Pair files exported from NimbleScan (Roche NimbleGen, 2010) were imported into the Bioconductor statistical environment (Gentleman et al., 2004). Sample-dependent methylated DNA immunoprecipitation (MeDIP) enrichments were estimated for each probe by fitting a fixed linear model accounting for array, dye, and sample effects to the data using the limma package (Smyth, 2004). The following statistical contrasts were then fit: B73 seedling IP sample vs. B73 seedling genomic DNA control (input), B73 embryo IP vs. input, B73 endosperm IP vs. input, B73 tassel IP vs. input, Mo17 seedling IP vs. input, Mo17 embryo IP vs. input, Mo17 endosperm IP vs. input, and Mo17 tassel IP vs. input. Four between-genotype statistical contrasts were also fit: Mo17 seedling vs. B73 seedling, Mo17 embryo vs. B73 embryo, Mo17 endosperm vs. B73 endosperm, and Mo17 tassel vs. B73 tassel. Finally, between-tissue statistical contrasts were developed for each genotype individually: embryo vs. leaf, embryo vs. tassel, endosperm vs. embryo, endosperm vs. leaf, endosperm vs. tassel, and leaf vs. tassel. Moderated *t*-statistics and the log-odds score for differential MeDIP enrichment was computed by empirical Bayes shrinkage of the standard errors with the false discovery rate controlled to 0.05. Results were formatted for the Integrative Genomics Viewer (IGV) (Robinson et al., 2011) for downstream analysis. Microarray results

were deposited with the National Center for Biotechnology Information GEO under accessions as described in Supplemental Table S1. Data tracks formatted for the IGV are available from http://genomics.tacc.utexas.edu/data/tissue_methylation_variation/.

Analysis of Variable Methylation

To identify segments showing differential methylation for all contrasts, the DNAcopy algorithm (Venkatraman and Olshen, 2007) was used on 1,088,517 Mo17 unique probes in the B73 vs. Mo17 relative methylation linear model results for all four tissues. Resulting segments were defined as differentially methylated regions (DMRs) if the segment mean of methylation values showed at least a twofold change (less than -1 or greater than 1 on the log₂ scale). These defined segments were used to assess methylation states in other contrasts. Values were scaled for all eight samples as $[x - \text{average}(y,z)/\text{maximum}(y,z) - \text{average}(y,z)]$ in which x is the unscaled sample value and y and z are the unscaled values that initially used in the discovery of the DMR. To validate similar methylation states within other contrasts, the segment mean of methylation values must show a 25% change in relative methylation state.

RNA Sequencing Analysis

An RNA sequencing (RNA-seq) analysis of all tissues described above was performed. Ribonucleic acid (RNA) isolated from three biological replicates of each sample was prepared for sequencing at the University of Minnesota BioMedical Genomics Center in accordance with the TruSeq library creation protocol (Illumina, 2012b). Samples were sequenced on the HiSeq 2000 (Illumina) developing 6 to 17 million reads per replicate. Raw reads were filtered to eliminate poor quality reads using CASAVA (Illumina, 2012a.). Transcript abundance was calculated by mapping reads to the maize reference genome (B73_RefGen_v2 [Maize Genome Sequencing Project, 2011]) using TopHat (Trapnell et al., 2009). A high degree of correlation between replicates was observed ($r > 0.98$). Reads per kilobase exon per million reads (RPKM) values were developed using “BAM to Counts” across the exon space of the maize genome reference working gene set (ZmB73_5a) within the iPlant Discovery Environment (www.iplantcollaborative.org).

Quantitative Real Time Polymerase Chain Reaction

Quantitative real time polymerase chain reaction (qPCR) validation of DMRs was adapted from Eichten et al. (2011). Briefly, primers were designed to amplify regions of 18 tDMR candidates (Supplemental Table S2). Sample DNA was digested with the methyl-sensitive restriction enzyme *Msp*JI (New England Biolabs) along with glycerol mock digests. The difference between digest threshold cycle [C(t)] and mock C(t) was calculated for each sample tested. As our selected enzymes target methylated cytosines, higher methylation leads to increased digestion and subsequently longer C(t) times.

Table 1. De novo discovery of differentially methylated regions (DMRs).

Contrast type	Contrast	First member of contrast high methylation	First member of contrast low methylation	Total	Total DMRs in contrast type
Genotype	Endosperm Mo17 vs. B73	159	344	503	2384
	Embryo Mo17 vs. B73	315	262	577	
	Leaf Mo17 vs. B73	378	358	736	
	Tassel Mo17 vs. B73	362	206	568	
Tissue	B73 embryo vs. leaf	16	0	16	600
	B73 embryo vs. tassel	27	51	78	
	B73 endosperm vs. embryo	86	59	145	
	B73 endosperm vs. leaf	42	27	69	
	B73 endosperm vs. tassel	33	63	96	
	B73 leaf vs. tassel	13	28	41	
	Mo17 embryo vs. leaf	17	4	21	
	Mo17 embryo vs. tassel	2	1	3	
	Mo17 endosperm vs. embryo	5	40	45	
	Mo17 endosperm vs. leaf	44	35	79	
	Mo17 endosperm vs. tassel	2	0	2	
	Mo17 leaf vs. tassel	3	2	5	

Results

The methylation state of 14-d seedling leaf, tassel, 14 d after pollination (DAP) embryo, and 14 DAP endosperm DNA from B73 and Mo17 inbred lines was assessed by MeDIP followed by hybridization to a microarray platform (see Methods). The array platform includes long oligonucleotide probes placed ~200 bp apart on low-copy sequences. The analysis of the signal ratio between immunoprecipitated DNA and a nonenriched control allows for the assessment of methylation enrichment across the maize genome (B73_RefGen_v2). To prevent aberrant signal from multicopy sequences as well as sequences absent from the Mo17 genome, all data was filtered to probes with only one unique copy in the B73 RefGen_v2 assembly and probes known to hybridize with similar efficiency in both B73 and Mo17 inbred lines (Eichten et al., 2011). The resulting 1,088,820 single-copy probes were used for a linear model based analysis that allowed for the isolation of genotype and DNA methylation effects. A linear model was developed to estimate average probe ratios for 12 tissue comparisons and four genotype comparisons (Table 1).

One approach to assessing the variation in DNA methylation among tissues and genotypes is to perform hierarchical clustering. Clustering of hypothetical single nucleotide polymorphism (SNP) data (Fig. 1A) or actual transcriptome (Fig. 1B) data across the four complex tissues illustrates the expectation for a genetic variation (such as SNPs) or primarily among tissues (as expected for transcriptomes). The clustering of DNA methylation data (Fig. 1C) reveals more substantial effects from genotype rather than tissue. Although most variation occurs between genotypes, there are some examples where tissue-specific methylation signals appear. The hierarchical clustering reveals that DNA methylation changes during development are limited relative to the differences between genotypes. We proceeded to assess

common differences among genotypes throughout development and subsequently to search for the rare examples of tissue-specific methylation variation within the sampled tissues.

Numerous Examples of Conserved Methylation Differences Found between Inbred Lines

There is widespread interest in understanding how DNA methylation patterns vary among individuals of the same species. We assessed whether different developmental stages would exhibit similar differences between two maize inbreds, B73 and Mo17. Differentially methylated regions between the two genotype DMRs (gDMRs) were identified in each tissue by using DNAcopy (Venkatraman and Olshen, 2007) to identify multiple adjacent probes with significant variation between the genotypes (Fig. 2A). Over 500 gDMRs with at least a twofold methylation difference were found between B73 and Mo17 in each of the four tissues that were assessed. There are similar proportions of gDMRs with higher methylation each of the two genotypes; 49% have higher methylation in B73 compared to Mo17 (Fig. 2A). While the number of gDMRs between B73 and Mo17 in each tissue was different we found that many of the regions had very similar differential methylation in all tissues profiled (Fig. 2B). The variation in actual number of gDMRs identified in each tissue is due to gDMRs that are near the statistical cutoffs in some tissues but pass all filters in other tissues. These results indicate that there are hundreds of gDMRs showing similar methylation variation between B73 and Mo17 in leaf, embryo, tassel, and endosperm tissue (Fig. 2B).

Few Tissue-Specific DNA Methylation Variants

It has been postulated that DNA methylation may play a role in proper regulation of development (Bird, 1997; Richards, 1997). To examine the impact of DNA methylation on development, an analysis of tissue-specific

DNA methylation was performed in four developmentally unique tissues. Six tissue contrasts were developed between the four tissues (seedling, embryo, tassel, and endosperm) for both B73 and Mo17 tissues. Using the same criteria used to discover gDMRs, substantially fewer tDMRs were discovered in tissue contrasts compared to genotype contrasts (Fig. 2C,D; Table 1). There were four times fewer tDMRs than gDMRs. Genotype DMRs often exhibit larger differences in methylation levels (median segment difference = 1.575) than tissue DMRs (median = 1.310) (Supplemental Fig. S1). A large portion of the DMRs (70% in B73 and 81% in Mo17) were identified in contrasts of endosperm with the other tissues.

Although there are significantly fewer tDMRs present between maize tissues, it is possible that some tDMRs play important roles in developmental regulation. Several filtering criteria were used to identify tissue-specific DMRs with potential functional roles in plant development. First, any tDMR that impacts development would be expected to show similar patterns in both genotypes if it plays a major role in proper development. Second, tDMRs involved in developmental gene regulation should be located near a gene that shows developmental variation for expression levels that is correlated with DNA methylation state.

We assessed whether tDMRs that were identified in a contrast of two tissues in one genotype had a conserved pattern in the same tissues of the other genotype (Fig. 2E). Only 115 (19%) of all 600 tDMRs showed similar DNA methylation patterns in both B73 and Mo17 (Fig. 3A; Supplemental Table S3) suggesting that the majority of tissue-specific DMRs appear to be inbred specific and are most likely not involved in important developmental regulation. The 115 examples of tissue-specific DMRs that are conserved in B73 and Mo17 include 12 examples of regions that were identified in multiple tissue contrasts resulting in a set of 103 unique tDMRs (Supplemental Table S3). The DNA methylation state in all four tissues was assessed for each of the 103 conserved tDMRs using hierarchical clustering (Fig. 2F). For both genotypes, the endosperm was most distinct relative to the other tissues. Nearly 70% of the 103 conserved tDMRs were identified in a contrast of endosperm with one of the vegetative tissues. There are smaller distinct clusters of genes for which leaf and tassel tissues show similar methylation levels compared to endosperm and embryo. Overall, there are many examples of tissue DMRs that appear to show a unique DNA methylation level in a single tissue while showing a uniform pattern in the remaining tissues (Fig. 2F; Supplemental Fig. S2).

Expression Variation of Genes near Tissue-Specific Differentially Methylated Regions

The 103 DMRs among maize tissues that were consistent in both B73 and Mo17 were further characterized to assess whether they were associated with tissue-specific expression of nearby genes. Transcription levels of maize genes were assessed using RNA-seq in the same tissues that were

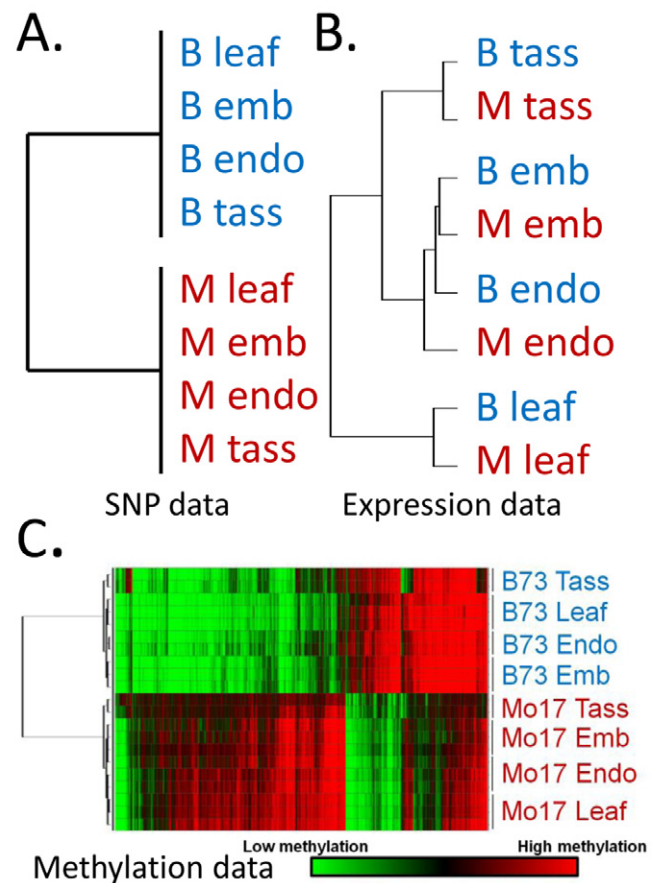


Figure 1. Hierarchical clustering of (A) single nucleotide polymorphism (SNP), (B) RNA sequencing (RNA-seq), and (C) DNA methylation levels in four tissues of B73 and Mo17. The clustering in (A) is an artificial plot that is based on the assumption that different tissues of the same plants will contain the same genotype. The clustering in (B) is based on two to three biological replicates of RNA-seq data for each of the eight tissue-genotype combinations. In (C), the DNA methylation profiles from MeDIP-chip profiling of the three biological replicates of each of the eight tissue-genotype combinations are used for hierarchical clustering (Ward's using Euclidean distance). Only probes with a significant difference in at least one of the tissue or genotype contrasts were used for clustering. The heatmap indicates high (red) or low (green) DNA methylation levels. B, B73; emb, embryo; endo, endosperm; M, Mo17; tass, tassel.

used for DNA methylation profiling (6–17 million reads per sample). There are 57 genes that were located within 5 kb of the tDMRs and are expressed in at least one of the tissues (RPKM > 0.1; Supplemental Table S4). The expression levels for 34 of these 57 genes near the tissue-specific DMRs exhibit a twofold or greater change in expression when comparing the two tissues used to discover the nearby tDMR. These 34 genes include 14 examples in which DNA methylation was negatively correlated with transcript abundance, as expected, and another 20 examples in which the transcript abundance and DNA methylation were positively correlated. Methyl-dependent restriction enzyme digests followed by qPCR were used to provide a validation for tissue-specific variation in DNA

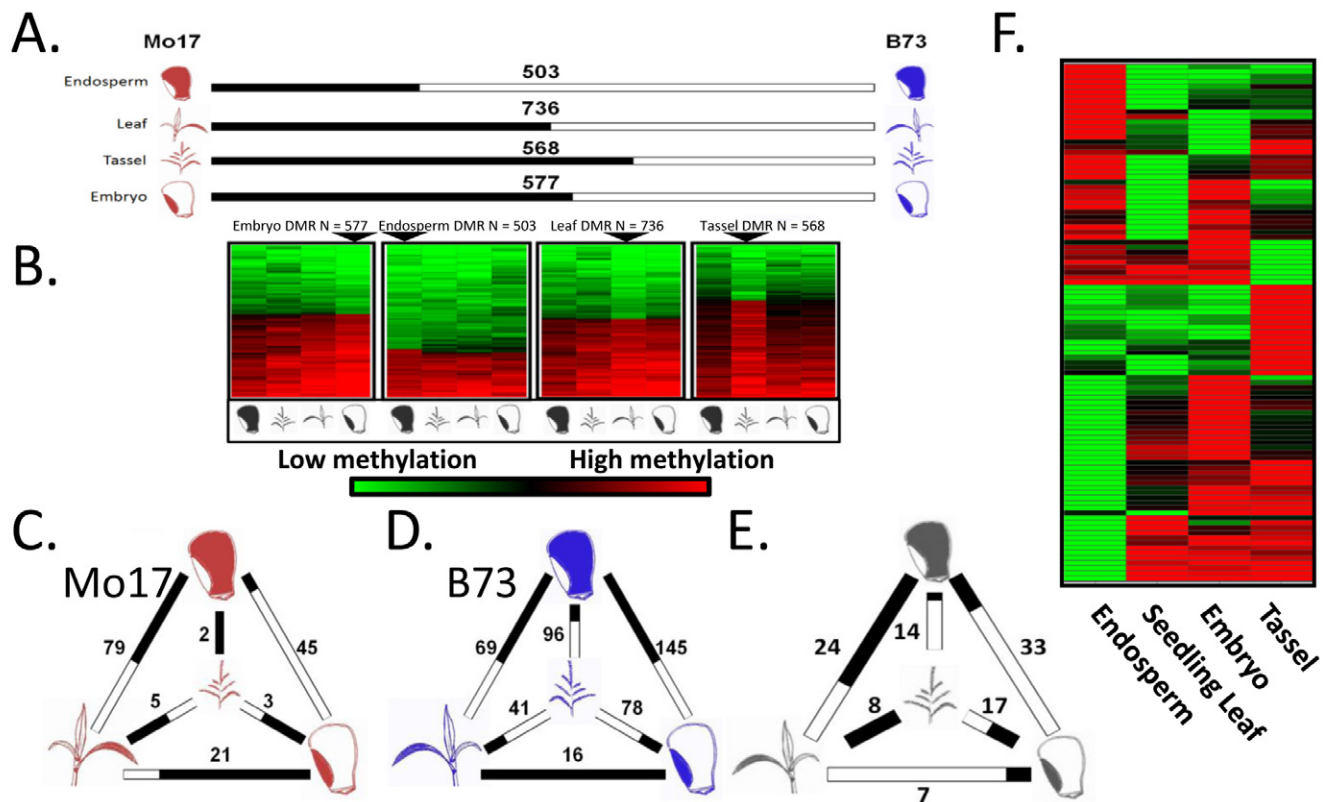


Figure 2. Discovery of differentially methylated regions (DMRs) among genotypes or tissues. (A) Genotype DMRs (gDMRs) were identified by contrasting each tissue of B73 and Mo17. The number above each bar indicates the total number of gDMRs and the shading of the bar indicates the proportion that are more highly methylated in each direction (the dark portion of the bar indicates the portion that are more highly methylated in Mo17). (B) Comparison of DNA methylation variation between genotypes in all four tissues. The gDMRs discovered in each of the four tissues (tissue used for discovery indicated above each plot) were used to perform hierarchical clustering (Ward's using Euclidean distance) of the DNA methylation levels of the same regions in all four genotypes. These plots provide evidence that regions of variable DNA methylation discovered in any one tissue exhibit reproducible genotype variation in the other three tissues. (C) The tissue DMRs (tDMRs) were separately identified in B73, Mo17 (D), and those conserved between the two genotypes (E). The numbers by each contrast indicate the total number and the shading of bars indicates the portion with higher methylation in each tissue. (F) Hierarchical clustering (Ward's using Euclidean distance; colored thresholds -2 and 2) of normalized tissue values for 103 nonredundant, genotype-conserved, tissue-specific DMRs.

methylation levels for 18 tDMRs (Fig. 3D; Supplemental Table S5). Eleven of 18 tDMRs tested selected from the 34 candidates were validated between the tissues used in the discovery of the tDMR.

A comparison of DNA methylation and transcript abundance in all tissues and genotypes identified six genes with a significant ($p < 0.05$) negative correlation between DNA methylation and expression state and four of the genes had an unexpected positive correlation between DNA methylation and expression (Fig. 3; Supplemental Table S4). For example, tDMR55 exhibits lower DNA methylation levels in tassel and is more highly expressed in this tissue (Fig. 3C). An example, tDMR51, displays higher DNA methylation levels that are correlated with higher expression. It is worth noting that many genes show variable tissue-specific expression patterns and we might expect some number of false positives when assessing expression patterns for any 103 random genes simply due to the frequency of tissue-specific variation. Several of the 10 correlated genes are similar ($e < 0.01$) to *Arabidopsis*

thaliana genes (Supplemental Table S6). The genes with a negative correlation between DNA methylation and expression include a MYB-domain gene similar to *ARR18*. There also was a putative histone acetylase with a positive correlation between DNA methylation and gene expression.

Discussion

Epigenetic memory likely occurs during both mitosis and meiosis. Our experiment allows us to examine the level of variation for DNA methylation, a chromatin modification often associated with epigenetic memory. We find more evidence for variation in DNA methylation patterns among genotypes than among developmental stages (Fig. 1). This suggests that DNA methylation may play a more substantial role in differentiating individuals of a species than in memory of developmental expression differences. Although a large number of gene expression changes occur as tissues develop within a plant, there is limited evidence for localized DNA methylation variation to play a role in this developmental process.

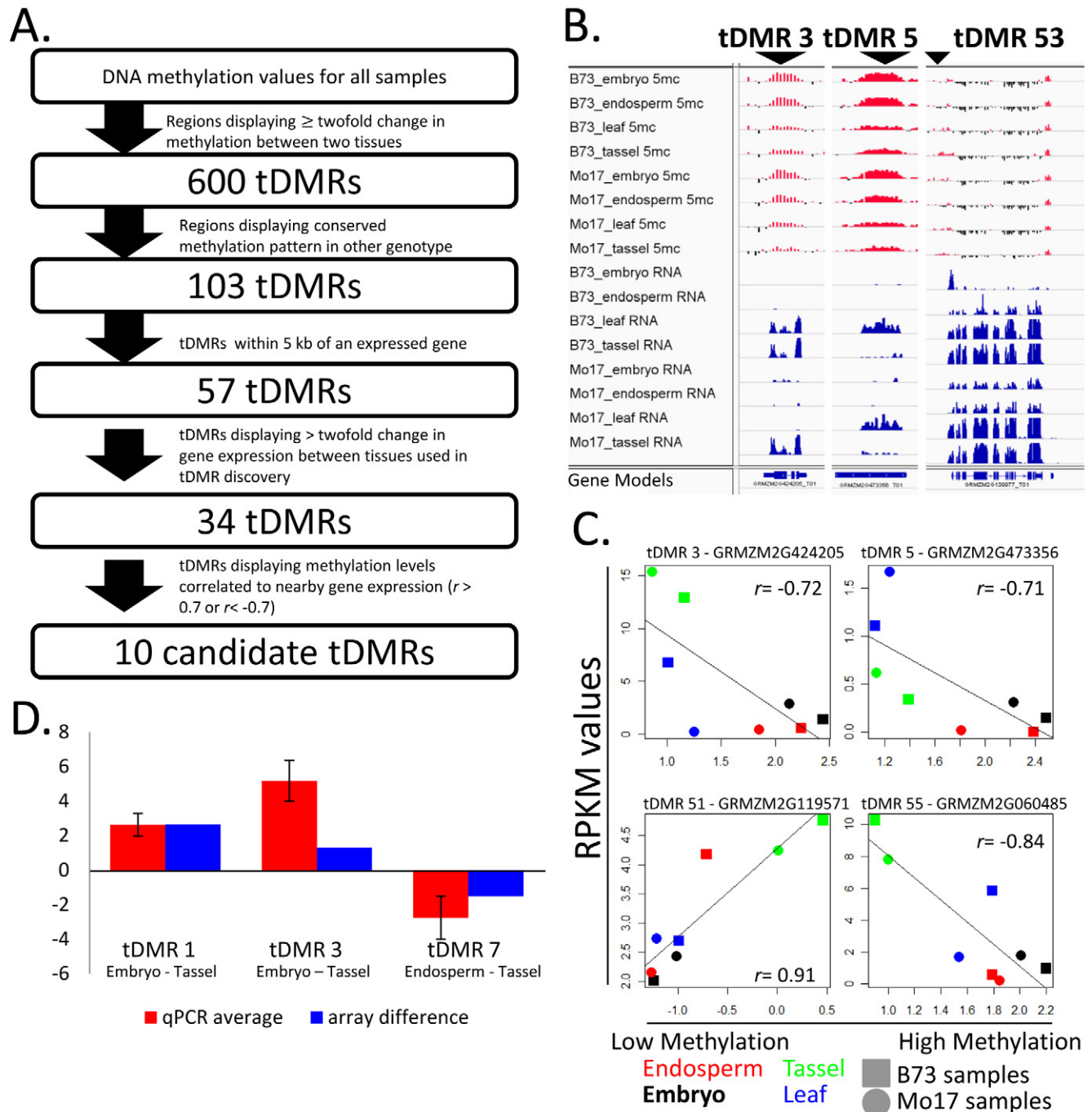


Figure 3. Relationship between tissue-specific DNA methylation and transcript abundance. (A) Flowchart describing sequential filtering of tissue-specific differentially methylated regions (tDMRs). (B) The level of DNA methylation (5mc is 5-methyl-cytosine) (red) from MeDIP-chip profiling and transcript abundance (blue) from RNA sequencing (RNA-seq) are shown for three tDMRs located near genes. (C) The correlation between DNA methylation and transcript abundance in the four tissues of both genotypes is shown for four tDMRs. (D) Example quantitative real time polymerase chain reaction (qPCR) validations of tDMRs. Array-based methylation values (blue) are the difference between the first and second tissues used in the tDMR discovery. Quantitative real time polymerase chain reaction values (red) are calculated as the difference between the first tissue (C(t)Digest – C(t)Mock) and the second (C(t)Digest – C(t)Mock).

Genotype-Specific Differentially Methylated Regions are Highly Conserved across Tissues

There is a growing effort to characterize DNA methylation differences among different individuals of the same species. Researchers are often confronted with decisions about the experimental design and are concerned that

developmental variation among two individuals may complicate the comparisons. Our data suggest that DNA methylation patterns are strikingly similar regardless of tissue assessed (Supplemental Fig. S3). In general, if we observe differences in DNA methylation among B73 and Mo17 in one tissue we saw similar variation in all other tissues for 84% of the genotype DMRs. In a previous

report we had identified 690 gDMRs among leaf tissue of B73 and Mo17. The combined analysis of all four tissues revealed a total of ~850 nonredundant gDMRs that meet all statistical criteria in at least one tissue and show conserved patterns of genotypic variation in all other tissues. Our data suggest that very similar DMRs would be found in any particular tissue but that the analysis of multiple tissues may allow for the most robust and complete set of genotype-specific DMRs. However, if necessary, it is possible to compare DNA methylation profiles of two individuals that vary in developmental morphology (such as wild and domesticated individuals) and still identify differences in DNA methylation due to genotype differences. Although the exact function of gDMRs among maize genotypes is still to be determined, the hundreds of regional differences in DNA methylation between B73 and Mo17 may provide a source of regulation explaining a portion of the differences between these two lines.

Few Tissue-Specific Differentially Methylated Regions Indicate Limited Role of DNA Methylation and Tissue Development

There has been evidence both for (Finnegan et al., 1996; Ronemus et al., 1996) and against (Zemach et al., 2010; Zhang et al., 2011) a broad role of DNA methylation in regulating gene expression during development in plants. In order for a methylation variant to play an important, predictable role in development, it would be expected that multiple different genotypes of maize would show similar tissue-specific DMRs. We found a limited number of tDMRs among the vegetative tissues and only a small number of these exhibit tDMRs in both genotypes. It would be expected that functionally relevant tDMRs would contribute to regulation of nearby genes. We found only 10 genes among the 57 located near tDMRs for which expression was correlated to the tDMRs methylation state (Fig. 3; Supplemental Table S4). These include six examples of negative correlation between DNA methylation and expression and four examples of positive correlation. We would expect that DNA methylation would show a negative correlation to gene expression as often methylation is associated with nearby gene silencing. The observed positive correlation of four tDMRs was surprising. It is possible that DNA methylation can be positively associated with gene expression. Studies of imprinted genes in *Arabidopsis thaliana* have identified examples where demethylation of certain imprinted genes is required for the gene to be silenced (Hsieh et al., 2009). From this, there may be certain genes within the genome that are not transcriptionally silenced unless demethylated. Alternatively, the existence of similar numbers of genes with positive and negative correlations with DNA methylation levels could suggest a lack of causation. Many genes exhibit tissue specific variation and we might expect some examples of correlated patterns in any set of 103 genes chosen by chance.

This study has provided evidence that DNA methylation does not show significant variation between tissues. Similar analyses of methylation across tissues

within plant systems show minimal tissue-specific methylation variation indicating a minimal influence of DNA methylation perturbation on development (Zhang et al., 2011). However, it is possible that tissue-specific DNA methylation variations are present that are beyond the scope of this study. By using an array-based technology to assess DNA methylation, we are unable to identify methylation variants smaller than 200 bp. Because of this, any tDMR smaller than our fragment size limits may go undetected in this study. It is also possible that tDMRs may go undetected given the strict filtering criteria used in this study. For example, requiring a twofold difference in DNA methylation levels would exclude the discovery of many of the allele-specific DNA methylation differences observed in endosperm. Beyond this, tissues selected in this study contain a variety of cell types that may display altered DNA methylation patterns but could be masked due to sampling multiple cell types at once within each tissue. Many development studies have shown that an increased resolution of tissue development allowed for a much greater capacity to identify tissue-specific differences in gene expression (Li et al., 2010). The same may indeed hold true for epigenetic studies.

Conclusions

Much debate has centered on the role of epigenetic regulation in tissue specification (Richards, 1997). Given the complexities of cellular and gene regulation required to correctly develop into a terminal tissue, it is possible that reversible epigenetic regulation of genes could provide a mechanism for gene expression variation during cellular differentiation. To investigate this possibility, we performed genomewide DNA methylation assessment across four distinct tissues of maize in two separate inbred lines. Although many conserved methylation variants could be identified across genotypes (gDMRs), very few tDMRs were identified. Tissue-specific DMRs initially identified exhibit poor agreement across genotypes as well as limited association with nearby gene expression variation. From these results, we conclude that there are very few, if any, tissue-specific methylation variants that provide developmental regulation of gene expression in maize.

Supplemental Information Available

Supplemental material is available at <http://www.crops.org/publications/tpg>.

Supplemental Table S1. Array design and replication information.

Supplemental Table S2. Tissue-specific differentially methylated region (tDMR) candidate quantitative real time polymerase chain reaction (qPCR) primers.

Supplemental Table S3. All tissue-specific differentially methylated regions (DMRs) and related filtering data.

Supplemental Table S4. Methylation levels for candidate tissue-specific differentially methylated regions (tDMRs) and expression information for nearest gene.

Supplemental Table S5. Quantitative real time polymerase chain reaction (qPCR) validations of 22 tissue-specific differentially methylated region (tDMR) candidates.

Supplemental Table S6. Putative function analysis of 10 genes correlated to tissue-specific differentially methylated region (tDMR) state.

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